Reference 9

PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07D 311/86, 493/14 C12N 1/14, C12P 17/06 A61K 31/35 | A1

(11) International Publication Number: WO 92/16517

(43) International Publication Date: 1 October 1992 (01.10.92)

(21) International Application Number: PCT/GB92/00526

(22) International Filing Date: 23 March 1992 (23.03.92)

(30) Priority data:

9106184.6	22 March 1991 (22.03.91)	GB
9111736.6	31 May 1991 (31.05.91)	GB
9116593.6	1 August 1991 (01.08.91)	GB
9205301.6	11 March 1992 (11.03.92)	GB

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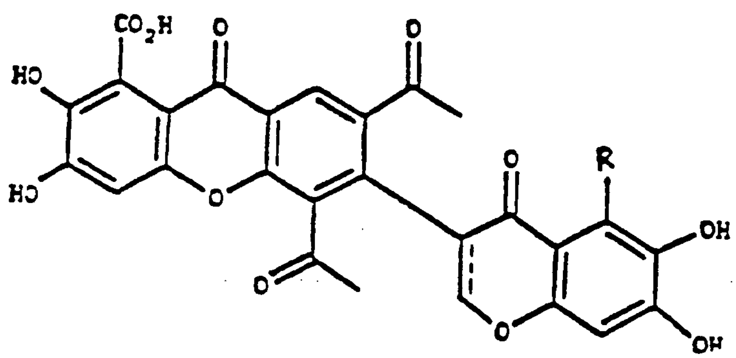
(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB, GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: PHARMACEUTICAL XANTHONE DERIVATIVES



(I)

(57) Abstract

A xanthone derivative of formula (I), wherein ---- is a single bond and R is -COOH or ---- is a double bond and R is H or COOH, and pharmaceutically and veterinarily acceptable salts, esters and ethers thereof, are useful as CD4 binding agents and inhibitors of protein kinase C and collagenase.

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PHARMACEUTICAL XANTHONE DERIVATIVES

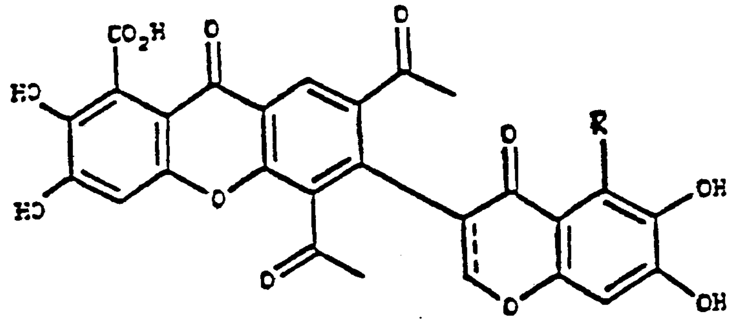
The present invention relates to compounds useful as CD4 binding agents and as inhibitors of the enzyme Protein Kinase C (PKC) and of collagenase, to the preparation of the compounds and to pharmaceutical and veterinary compositions containing them.

CD4 is a cell surface glycoprotein expressed on those T lymphocytes which recognise antigen bound to class II MHC molecules. Inhibition of class II MHC-CD4 interactions will block antibody responses, mixed lymphocyte reactions and other immune responses involving $CD4^+$ T lymphocytes. These responses are important in pathological conditions including autoimmunity, organ graft rejection, allergy and graft versus host disease. CD4 is also the cellular receptor for human immunodeficiency virus (HIV).

PKC is critically involved in activation of T lymphocytes. Interaction of the T cell antigen receptor with its ligand causes increased turnover of phosphatidylinositol lipids and the generation of diacylglycerol (DAG) which activates PKC.

Collagenase is a member of the connective tissue metalloproteinase family of enzymes. Collagenases control the turnover, remodelling and degradation of collagen in tissue. Collagen breakdown occurs in several pathological conditions including arthritis, tumour metastasis, periodontal disease, corneal ulceration and excessive bone or skin collagen degradation.

We have now discovered that fermentation of a nutrient medium with a strain of the fungus Penicillium glabrum produces novel xanthone derivatives of formula 1, which may be recovered and purified. These compounds are CD4-binding agents and inhibitors of PKC and collagenase. Accordingly, the present invention provides a xanthone derivative of formula 1:



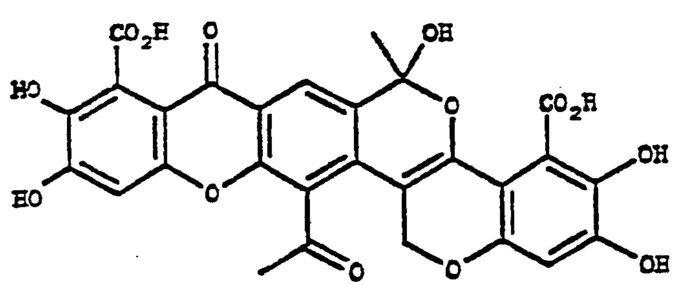
wherein ---- is a single bond and R is -COOH or ---- is a double bond and R is H or -COOH (hereinafter referred to as the present compounds); and pharmaceutically and veterinarily acceptable salts, esters and ethers thereof. Preferred compounds of the invention are:

2,4-diacetyl-3-(2',3'-dihydro-5'-carboxy-6',7'-dihydroxychromon-3'-yl)-6,7-dihydroxyxanthone-8-carboxylic acid (compound [A]);

2,4-diacetyl-3-(5'-carboxy-6',7'-dihydroxychromon-3'-yl)-6,7-dihydroxyxanthone-8-carboxylic acid (compound [B]) and the permethylated derivative thereof; and

2,4-diacetyl-3-(6',7'-dihydroxychromon-3'-yl)-6,7-dihydroxyxanthone-8-carboxylic acid (compound [C]).

The derivative of formula 1 in which ---- is a single bond and R is -COOH, compound [A], may also be represented by an isomeric structure of formula 2 below. This isomer falls within the scope of the invention too. In the present specification, therefore, references to the derivative of formula 1 in which ---- is a single bond and R is -COOH encompass the isomer of formula 2 as well.



The present compounds have been isolated from a microorganism which we have designated strain X8063 and which has been identified as a strain of the fungus Penicillium glabrum on the basis of the following morphological data:

Fungal strain X8063 was incubated for 7 days at each of 5°C, 25°C and 30°C in the following growth media (from PITT, J.I., 1979; The Genus Penicillium and its Teleomorphic States Eupenicillium and Talaromyces. London: Academic Press.): Czapek's agar with yeast extract (CzYa; composition per litre of distilled water: sucrose, 30g; yeast extract, 5g; agar 20g; KH_2PO_4, 1g; $NaNO_3$, 0.3g; $MgSO_4.7H_2O$, 0.05g; KCl, 0.05g; $FeSO_4.7H_2O$, 0.001g); malt extract agar with glucose and peptone (MEA; composition per litre of distilled water: malt extract; 20g; glucose, 20g; Bacto-peptone, 1g; agar, 20g); glycerol nitrate agar (G25N; composition per 750 ml of distilled water: yeast extract, 3.7g; glycerol, 250g; KH_2PO_4, 0.75g; $NaNO_3$, 0.225g; $MgSO_4.7H_2O$, 37.5mg; KCl, 37.5mg; $FeSO_4.7H_2O$, 0.75mg).

The microscopic characteristics were as follows: Conidiophores borne from surface hyphae. Stipes of the conidiophores up to 200 μm long and 4-5 μm wide, vesiculate (vesicles 6-7 μm wide) with smooth walls. Verticils borne terminally (never subterminal), monoverticillate, composed of 10 to 16 appressed phialides. Phialides ampulliform (10)-12-13 x 4-5 μm. Conidia spheroidal, 3.5-4.5 μm diameter, with faintly warted/rugulose walls. Conidia borne in long well defined columns.

The gross colony morphology on each growth medium was as follows (colours are coded according to the British Standards Institute publication BS381C:1980; Colours for identification, coding and special purposes):

CzYa at 25°: Colonies, 31 to 40 mm diameter, plane with radial sulcation. Texture velutinous, low. Margin immersed, entire and extending approximately 1 mm. Mycelium white to light beige (366) with a plane, non-

sporulating, 2-4 mm periphery. Central region white, but showing in places a slight greenish tint, conidiogenesis poor. Exudate and sclerotia absent. Pale clear yellow diffusing pigment produced. Diffusion zone approximately 50mm in diameter. Reverse concolourous with surface mycelium, a yellowish pale cream (352).

MEA at 25°: Colonies, 30 to 31 mm diameter, plane, lacking radial sulcation. Texture velutinous, low. Margin not immersed, entire. Aerial mycelium very sparse, colours due to maturing spore mass; outer region (5 mm margin) approximating pale cream (352) to light straw (384) but slightly more yellowish, central region approximating light grey (631) to aircraft grey (693) but slightly more bluish. Conidiogenesis moderate. Exudate and sclerotia absent. Diffusing pigment produced in a zone approximately 45 mm in diameter, coloured a clear bright pale yellow. Reverse darkening towards the centre ranging from pale cream (352) to dark earth (450).

G25N at 25°: Colonies, 12 to 13 mm diameter, plane with radial sulcation. Texture velutinous, low. Margin immersed and entire. Outer region (1-2 mm) colourless to white. Central region white to very pale yellow. Conidiogenesis very poor and lacking distinct spore mass colour. Exudate and sclerotia absent. Diffusing pigment absent. Reverse ranging from white to pale bright yellow (slightly paler than canary yellow, 309).

CzYa at 5°: Slight growth, colonies 3 to 5 mm diameter.

MEA at 5°: Micro-colonies to slight growth, 3 to 4 mm diameter.

G25N at 5°: No growth.

CzYa, MEA and G25N at 30°: No growth.

The production of penicillate conidophores clearly places strain X8063 in the genus *Penicillium*. From the other microscopic and gross morphological features of strain X8063 described above, and following the taxonomic

scheme in Pitt, J.I., 1979 - The Genus Penicillium and its Teleomorphic States Eupenicillium and Talaromyces: London, Academic Press, X8063 may be best classified as a strain of Penicillium glabrum (Wehmer) Westling (a widely used synonym is P. frequentans Westling).

The strain X8063 was isolated from a soil sample collected from Femes, Lanzarote in 1987 and was deposited under the Budapest Treaty at the Commonwealth Mycological Institute, Kew, Richmond, Surrey, UK on 13 December 1989 under accession number CMI 336456.

The above description is illustrative of a strain of Penicillium glabrum which can be employed in the production of the present compounds. However, the present invention also embraces the use of mutants of strain X8063 which produce the compounds of the invention. For example, those mutants which are obtained by natural selection or those produced by mutating agents including ionising radiation such as ultraviolet irradiation, or chemical mutagens such as nitrosoguanidine or the like treatments, are also included within the ambit of this invention.

The present invention further provides a process for the preparation of a xanthone derivative of formula 1 or a pharmaceutically or veterinarily acceptable salt, ester or ether thereof, which process comprises (i) fermenting, in a source of carbon, nitrogen and inorganic salts, fungal strain X8063 (CMI 336456) or a mutant thereof which produces a said xanthone derivative; (ii) isolating a said xanthone derivative from the fermentation medium; and (iii) if desired, converting the said xanthone derivative into a pharmaceutically or veterinarily acceptable salt, ester or ether thereof.

The present compounds are typically produced during the aerobic fermentation of an aqueous nutrient medium under conditions described hereinafter, with a producing strain of Pencillium glabrum, X8063, or a producing mutant strain of X8063. Aqueous media such as those used for the

production of many antibiotic substances are suitable. Such nutrient media contain sources of carbon and nitrogen assimilable by the microorganism. If desired inorganic salts may be added, generally at low levels. In addition, the fermentation media may contain traces of metals necessary for the growth of the microorganisms, and production of the desired compound. These are usually present in sufficient concentrations in the complex sources of carbon and nitrogen, which may be used as nutrient sources, but can, of course, be added separately to the medium if desired.

The invention therefore further provides a biologically pure culture of fungal strain X8063 or of a mutant thereof which produces a xanthone derivative of formula 1. Such cultures are substantially free from other microorganisms. The invention also provides a process for fermenting fungal strain X8063 or a mutant thereof which produces the xanthone derivative of formula 1, which process comprises fermenting strain X8063 or a said mutant thereof in a source of carbon, nitrogen and inorganic salts.

Assimilable sources of carbon, nitrogen and minerals may be provided by either simple or complex nutrients. Sources of carbon will generally include glucose, maltose, starch, glycerol, molasses, dextrin, lactose, sucrose, fructose, carboxylic acids, amino acids, glycerides, alcohols, alkanes and vegetable oils. Sources of carbon will generally comprise from 0.5 to 10% by weight of the fermentation medium.

Sources of nitrogen will generally include soya bean meal, corn steep liquors, distillers' solubles, yeast extracts, cottonseed meal, peptones, ground nut meal, malt extract, molasses, casein, amino acid mixtures, ammonia (gas or solution), ammonium salts or nitrates. Urea and other amides may also be used. Sources of nitrogen will generally comprise from 0.1 to 10% by weight of the

fermentation medium.

Nutrient mineral salts which may be incorporated into the culture medium include the generally used salts capable of yielding sodium, potassium, ammonium, iron, magnesium, zinc, nickel, cobalt, manganese, vanadium, chromium, calcium, copper, molybdenum, boron, phosphate, sulphate, chloride and carbonate ions.

An antifoam may be present to control excessive foaming and added at intervals as required.

The fermentation using Penicillium glabrum can be conducted at temperatures ranging from 20°C to 40°C, preferably 24-30°C. For optimal results, it is most convenient to conduct these fermentations at a temperature in the range 24-26°C. The starting pH of the nutrient medium suitable for producing the compounds can vary from 5.0 to 8.5 with a preferred range of from 5.5 to 7.5.

Small scale fermentations are conveniently carried out by placing suitable quantities of nutrient medium in a flask by known sterile techniques, inoculating the flask with either spores or vegetative cellular growth of Penicillium glabrum, loosely stoppering the flask with cotton wool, and permitting the fermentation to proceed in a constant room temperature of about 25°C on a rotary shaker at from 95 to 300 rpm for 2 to 10 days. The fermentation may also be conducted in static culture on liquid or semi-solid medium.

For larger scale work, it is preferable to conduct the fermentation in suitable tanks provided with an agitator and a means of aerating the fermentation medium. The nutrient medium is made up in the tank after sterilization and is inoculated with a source of vegetative cellular growth of Pencillium glabrum. The fermentation is allowed to continue for from 1 to 8 days while agitating and/or aerating the nutrient medium at a temperature in the range 24°C to 37°C. The degree of aeration is dependent upon several factors such as the size of the fermenter and

agitation speed. Generally the larger scale fermentations are agitated at about 95 to 500 rpm and aerations of about 0.5 to 1.5 VVM (volumes of air per volume of medium per minute).

The present compounds are found primarily in the liquor of the fermentation of strain X8063 and may be removed and separated as described below.

The separation of the present compounds from the whole fermentation broth and their recovery is carried out by solvent extraction followed by application of chromatographic fractionations with various chromatographic techniques and solvent systems. The present compounds in pure form have thus been isolated in this way.

The present compounds are acidic and are soluble in neutral and alkaline aqueous solvents. In their unionised forms the compounds are soluble in polar organic solvents such as dimethyl sulphoxide. When impure, the uncharged forms of the compounds are soluble in a wider range of organic solvents such as dichloromethane and ethyl acetate. Thus, in one recovery method, the whole fermentation broth is acidified to pH 3 and combined with a water-immiscible organic solvent such as ethyl acetate. Generally, several extractions are required to achieve maximal recovery. The solvent removes the desired compounds, but also other substances.

As the desired compounds are acids they can be purified further by back-extracting the water-immiscible solvent extract with a buffered alkaline aqueous solution of a salt such as ammonium acetate or sodium phosphate. In this process the present compounds are ionised and are extracted into the aqueous layer. The present compounds and other acidic compounds can then be recovered from the aqueous layer by acidifying it to pH 3 and re-extracting it with fresh water-immiscible organic solvent. This solvent extract is then concentrated under reduced pressure.

An alternative method of recovery of the desired

compounds from the liquor, particularly useful for large scale fermentations, is to acidify the liquor to pH 3 and pass the acidified liquor through a chromatography column filled with a porous hydrophobic resin such as Diaion HP20 (Mitsubishi Kasei Corp). The resin is then washed with an acidic aqueous solution such as an acetic acid solution and then eluted with a water-miscible organic solvent such as methanol. The methanol eluate is concentrated under reduced pressure before diluting it with ethyl acetate or another water-immiscible organic solvent and purifying the organic solvent solution by back-extraction with an alkaline aqueous buffer solution, re-acidification of the aqueous back extract, re-extraction into organic solvents and concentration, as described in the last paragraph.

The residue produced by either of these two extraction methods is then further purified by reverse phase high-pressure liquid chromatography (hplc) under acidic conditions on a column containing an adsorbent such as octadecyl silica. The column retains the desired compounds and other acidic impurities. It is eluted with a mixture of an acidic aqueous solution, such as acetic acid solution, and a water-miscible organic solvent, such as tetrahydrofuran (THF). As the proportion of the organic solvent in the mixture is increased the desired compounds are eluted after most of the impurities. Fractions of eluate are then typically concentrated and examined for the presence of the desired compounds by analytical hplc with photodiode array detection, as described in Examples 4, 6 and 10. Each of the compounds of the invention can then be individually identified from its uv/visible spectrum and retention time.

An eluate containing a desired compound is extracted with a water-immiscible organic solvent such as ethyl acetate and the desired compound recovered by evaporation of the extract. The desired compound is further purified by reverse phase hplc on an octadecyl silica column eluted

isocratically with mixtures of the same solvents used in the last hplc purification stage and recovered from the eluate by extraction in the same way.

The use of analytical hplc or other known techniques will afford purified compositions containing the desired compounds, the presence of which is determined by analysing the various chromatographic fractions for CD4 binding activity, PCK inhibitory activity and/or collagenase inhibitory activity or for physicochemical characteristics.

A xanthone derivative of formula 1 may be converted into a pharmaceutically or veterinarily acceptable salt, ether or ester. A xanthone derivative of formula 1 may therefore be converted into a per(C_1-C_6 alkylated) derivative thereof, for example a per(C_1-C_4 alkylated) derivative such as the permethylated or perethylated derivative thereof. Suitable salts include salts with alkali metals such as sodium and potassium, and ammonium salts.

Suitable ethers are branched or unbranched, saturated or unsaturated, substituted or unsubstituted C_1 to C_6 aliphatic ethers, typically C_1 to C_6 alkyl ethers. Preferred alkyl ethers are C_1 to C_4 alkyl ethers such as the methyl and ethyl ethers. Typically the ethers are peralkyl ethers. The ether groups may be substituted by hydroxy or halogen, for example fluorine, chlorine, bromine or iodine.

Such ethers may be prepared by treatment of the xanthone derivative with a diazoalkane such as diazomethane in a suitable inert solvent or by treatment with an appropriate alkyl halide, sulphonate ester or dialkyl sulphate in the presence of base. Suitable bases include alkali and alkaline earth metal hydroxides, tetraalkylammonium hydroxides and alkali and alkaline earth metal carbonates.

Suitable esters include esters formed with branched or unbranched, saturated or unsaturated, substituted or

unsubstituted C_1-C_6 alcohols. Methyl, ethyl and vinyl esters are typical examples. Such esters may be prepared in the same way as the ethers above or by treatment of the xanthone derivative with the appropriate alcohol in the presence of a suitable acid catalyst or activating agent.

The present compounds and their salts, esters and ethers have utility as CD4 binding agents or inhibitors of PKC or collagenase. They can block the physiological function of CD4 by inhibiting its interaction with MHC class II molecules. A human or animal, eg mammal, can therefore be treated by a method comprising administration of a therapeutically effective amount of a xanthone derivative of formula 1 or a pharmaceutically or veterinarily acceptable salt, ester or ether thereof.

A CD4 binding agent can selectively inhibit MHC class II-restricted responses. The present compounds and their salts, esters and ethers can therefore be used as immunosuppressants, especially in the treatment of autoimmune disease such as rheumatoid arthritis, systemic lupus erythematosus, diabetes mellitus, multiple sclerosis and primary biliary cirrhosis, graft versus host (GVH) disease and organ rejection. They can also be used in the treatment of allergic diseases such as asthma and inflammatory diseases such as inflammatory bowel disease. A CD4 binding agent may be effective in preventing the entry of HIV into cells and hence of therapeutic value as an anti-HIV agent.

The present compounds are active in an ELISA assay based on the interaction between soluble recombinant CD4 (sCD4 (V1 + V2); from E. coli) and the monoclonal antibody Leu3a (Becton-Dickenson, Oxford, England). Leu3a binds to an epitope on the V1 domain of CD4 as described by Merkenschlager et al (1990) (Merkenschlager, M., Buck, D., Beverley, P.C.L. and Sattentau, Q.J. (1990) "Functional epitope analysis of the CD4 molecule" J. Immunology 145 2839-2845). The present compounds bind to sCD4 and prevent

binding of Leu3a with IC_{50} values, calculated as the result of several experiments, of 2.5μM (compound [A]), 1.0μM (compound [B]) and 22μM (compound [C]). Tables 1 to 3 show the degree of inhibition observed at various concentrations of the present compounds.

TABLE 1: Inhibition of Binding of Leu 3a to soluble recombinant CD4 by compound [A]

Concentration of compound, $Mx10^6$	% Inhibition
0.4	26
0.8	35
1.6	40
3.1	54
6.2	59
12.5	69
25.0	79
50.0	85
100.0	85
200.0	87

TABLE 2: Inhibition of Binding of Leu 3a to soluble recombinant CD4 by compound [B]

Concentration of compound, $Mx10^6$	% Inhibition
0.3	24
0.7	43
1.4	61
2.7	75
6.4	84
10.9	96
21.8	100
43.5	102
87.0	102
174.0	103

TABLE 3: Inhibition of binding of Leu3a to soluble recombinant CD4 by compound [C]

Concentration of compound, M x 10^6	% inhibition
1.56	4
3.12	10
6.25	7
12.5	42
25.0	53
50.0	67
100.0	73
200.0	80

Compound [B] has also shown activity which correlates with its binding to CD4 in functional assays based on the inhibition of CD4-dependent T cell responses. It inhibits the CD4-dependent streptolysin-induced proliferative response of normal T lymphocytes isolated from blood with an IC_{50} of 9μm, as shown by the data in Table 4. It does not inhibit the mitogen-induced (PHA) response, which is dependent principally on CD2, at concentrations of up to 100μM.

TABLE 4. Inhibition of Streptolysin 0 (SLO)- and Phytohaemagglutinin (PHA)-Stimulated Proliferation of Human T-Cells by compound [B]

Concentration of compound, Mx10^6	% Inhibition of Lymphocyte Proliferation	
	SLO-Stimulated	PHA-Stimulated
0.5	12	-12
1.0	23	-22
5.0	29	-11
10.0	53	-3
20.0	71	-2

Compound [B] also inhibits the CD4-dependent mixed

lymphocyte reaction (MLR). The data shown in Table 5 demonstrate that compound [B] inhibits a two-way MLR with an IC_{50} of 7μM. There was no evidence for any cytostatic/cytotoxic effects of the compound on the CD4 T cell tumour line HSB-2 up to a concentration of 100μM in the same experiment.

TABLE 5: Inhibition of a mixed lymphocyte reaction (MLR) and proliferation of HSB-2 cells by compound [B]

Concentration of compound, Mx10^6	% Inhibition MLR	% Inhibition of HSB-2 cell proliferation
0.05	-5	-7
0.1	0	-2
0.5	-3	-8
1.0	3	-5
5.0	41	5
10.0	67	0
20.0	62	-5
50.0	97	3
100.0	100	-5

In their role as PKC inhibitors the present compounds and their salts, esters and ethers can be employed to alleviate a broad range of cancerous conditions. They therefore have utility as antitumour agents. They can therefore be used in a method of treating tumours such as breast, thyroid, colon, lung, skin and brain tumours, in particular tumours in which PKC is implicated as a causative agent.

The present compounds and their salts, esters and ethers can also be employed to alleviate inflammatory conditions and therefore have utility as anti-inflammatory agents. They can be used in a method of treating inflammatory conditions, for example asthma.

An inhibitor of PKC also inhibits T cell activation and can be used in the therapy of autoimmune diseases. The present compounds and their salts, esters and ethers therefore have utility in the therapy of autoimmune disease and can be used in a method of treating autoimmune disease such as systemic lupus erythematosus, myasthenia gravis and diabetes.

A PKC inhibitor also acts as an immunosuppressive agent. The present compounds and their salts, esters and ethers therefore have utility as immunosuppressive agents and can be used in a method of suppressing an immune response in a human or animal. For example, the present compounds and their salts, esters and ethers can be used in a method of preventing organ graft rejections.

The present compound is active in a PKC inhibition assay, as described in Example 12. According to this assay, the present compounds were found to inhibit the action of PKC purified from bovine brain with an IC_{50}, calculated as the result of several experiments, of about 2μM (compounds [A] and [B]) and of 6μM (compound [C]).

Collagenase plays a wide-ranging role in the pathology of disease. In its role as a collagenase inhibitor the present compounds and their salts, esters and ethers can be employed to alleviate conditions including rheumatoid arthritis and osteoarthritis, tumour metastasis, in periodontal disease, corneal ulceration, excessive skin or bone collagen degradation and other disorders.

The assay for collagenase inhibiting activity is based on the degradation of rat type I collagen gels by human collagenase as described by Harris and Vater (1982) (Harris, E.D. and Vater, C.A. (1982) in Methods in Enzymology (Cunningham, L.W. and Frederiksen, O.W., eds), Vol 82 part A, pp 423-452, Academic Press, New York).

The degree of lysis is estimated after staining residual substrate with Coomassie Brilliant Blue. Rat tail tendon type I collagen is partially purified by the method

described by Miller and Rhodes (182) (Miller, E.J. and Rhodes, R.K. (1982) in Methods in Enzymology (Cunningham, L.W. and Frederiksen, O.W., eds), Vol 82 part A, pp 33-64, Academic Press, New York) and human collagenase is derived from the conditioned medium of a human histiocytic lymphoma cell line (U937). Enzyme production is stimulated by the addition of PMA (phorbol myristate acetate) and the latent enzyme activated with trypsin.

In a collagenase inhibition assay as described above the present compounds were found to inhibit the action of human collagenase on rat type I collagen with an IC_{50} of 2μM (compound [A]) and 4μM (compound [B]). At a concentration of 25μM compound [C] caused 53% inhibition of collagenase. The following Tables 6 and 7 show the degree of inhibition observed at various concentrations:

TABLE 6: Degree of collagenase inhibition produced by compound [A]

Concentration of compound, $Mx10^6$	% Inhibition
0.4	46
1.6	49
6.25	71
25.0	87
100.0	94

TABLE 7: Degree of collagenase inhibition produced by compound [B]

Concentration of compound, $Mx10^6$	% Inhibition
0.5	0
1.1	3
2.2	29
4.3	52
8.7	77
17.4	85
34.7	81
69.4	78
138.9	79

The present compounds and their salts, esters and ethers can be administered in a variety of dosage forms, for example orally such as in the form of tablets, capsules, sugar- or film-coated tablets, liquid solutions or suspensions or parenterally, for example intramuscularly, intravenously or subcutaneously. The present compounds and their salts, esters and ethers may therefore by given by injection or infusion.

The dosage of one of the present compounds or of their salts, esters or ethers depends on a variety of factors including the age, weight and condition of the patient and the route of administration. Typically, however, the dosage adopted for each route of administration to adult humans is 0.001 to 10mg/kg, most commonly in the range of 0.01 to 5 mg/kg, body weight. Such a dosage may be given from 1 to 5 times daily. The present compounds and their salts, esters and ethers are non-toxic at therapeutic doses.

The present compounds and their salts, esters and ethers are formulated for use as pharmaceutical or veterinary compositions also comprising a pharmaceutically or veterinarily acceptable carrier or diluent. The

compositions are typically prepared following conventional methods and are administered in a pharmaceutically or veterinarily suitable form.

For example, the solid oral forms may contain, together with the active compound, diluents such as lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants such as silica, talc, stearic acid, magnesium or calcium stearate and/or polyethylene glycols; binding agents such as starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose, or polyvinyl pyrrolidone; disintegrating agents such as starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dye-stuffs; sweeteners; wetting agents such as lecithin, polysorbates, laurylsulphates. Such preparations may be manufactured in known manner, for example by means of mixing, granulating, tabletting, sugar coating, or film-coating processes.

Liquid dispersions for oral administration may be syrups, emulsions and suspensions. The syrups may contain as carrier, for example, saccharose or saccharose with glycerol and/or mannitol and/or sorbitol. In particular a syrup for diabetic patients can contain as carriers only products, for example sorbitol, which do not metabolise to glucose or which only metabolise a very small amount to glucose. The suspensions and the emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose or polyvinyl alcohol.

Suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier such as sterile water, olive oil, ethyl oleate, glycols such as propylene glycol, and, if desired, a suitable amount of lidocaine hydrochloride. Solutions for intravenous injection or infusion may contain a carrier, for example, sterile water which is generally Water for Injection. Preferably,

however, they may take the form of a sterile, aqueous, isotonic saline solution. Alternatively, a compound may be encapsulated within liposomes.

The following Examples illustrate the invention.

Example 1 - Culture of strain X8063 in Liquid Media (1)

Starting material of the strain X8063 was generated by suspending a mature slant culture, grown on PDA (2% dextrose, 15% agar, 0.4% potato extract), in 5 ml 10% aqueous glycerol. 1 ml of this suspension, in a 1.5 ml cryovial, comprises starting material, which was retrieved from storage at -135°C. A preculture was produced by aseptically placing 0.5 ml starting material in 20 ml nutrient solution S(1.5% glycerol, 1.5% soya bean peptone, 1% glucose, 0.5% malt extract, 0.3% NaCl, 0.1% $CaCO_3$, 0.1% Tween 80, 0.1% Junlon PW110, pH6) in an Erlenmeyer flask shaken at 240 rpm for 2 days at 25°C. After this period a further 20 ml of nutrient solution S was added and this secondary preculture was incubated at 25°C for 3 days in a rotary shaker at 240 rpm.

An intermediate culture was then generated by aseptically transferring a secondary preculture to 2.01 of nutrient solution S in a 31 stirred fermenter and incubating for 3 days at 25°C.

A production culture was generated by aseptically transferring an intermediate culture to a 751 stirred fermenter containing 501 of nutrient solution P (6.04% molasses. 0.339% casein enzymatic hydrolysate, 0.1% $CaCO_3$, 0.1% Tween 80, 0.006% sodium phytate, pH6) and incubating at 25°C. After 5 days the liquor was harvested for extraction of the product.

The strain X8063, also grew well in cultures incubated in tubes shaken at 240 rpm for 8 days at 25°C in nutrient solution A (2.35% sucrose, 0.97% MES buffer, 0.967% monosodium glutamate, 0.05% $MgSO_4.7H_2O$, 0.05% KCl,

0.0037% K_2HPO_4, 0.002% $CaCl_2.2H_2O$, 20 ml L^{-1} Vitamin mixture I, 5 ml L^{-1} trace element mixture II, 1 ml L^{-1} Tween 80).

Vitamin mixture consists of (mg L^{-1}): thiamine 25, riboflavin 25, pantothenic acid 25, niacin 25, pyridoxine 25, thiacetic acid 25, folic acid 2.5, biotin 2.5, cyanocobalamin 2.5, p-aminobenzoic acid 2.5, vitamin K_1 2.5.

Trace Element Mixture II consists of (per litre): 1 ml 1M H_2SO_4, 287 mg $ZnSO_4.7H_2O$, 223 mg $MnSO_4.4H_2O$, 125 mg $CuSO_4.5H_2O$, 83 mg KI, 62 mg H_3BO_3, 48 mg $NaMoO_4.2H_2O$, 48 mg $CoCl_2.6H_2O$.

<u>Example 2 - Extraction of CD4-binding activity from fermentation of the strain X8063</u>

A 501 fermentation of the strain X8063 prepared as in Example 1 was centrifuged and the mycelium discarded. The pH of the aqueous supernatant (401) was adjusted from 7.0 to 3.0 with glacial acetic acid and Diaion HP20 resin (4.01 by volume, Mitsubishi Kasei Corporation) was added with stirring and the mixture left to stand overnight. The resin was collected by filtration, washed with methanol/0,05 M aqueous acetic acid (1:1, 5.0 l) and then eluted with methanol (101). The methanol eluate was concentrated to a volume of approximately one litre by rotary evaporation and then diluted with 0.05 M aqueous acetic acid (51). This aqueous mixture was extracted with ethyl acetate (2x51) and the ethyl acetate extract concentrated to dryness to yield a brown gum (20.2g) which contained the CD4-binding compounds of interest.

<u>Example 3 - Purification of compound [A]</u>

A portion (4g) of the ethyl acetate extract prepared in Example 2 was dissolved in methanol (20ml) and diluted with 0.05M aqueous acetic acid (30ml). This solution was purified by preparative reverse phase hplc on a PrepPak cartridge column (1D 25 mm x length 100 mm, packed with

Nova-Pak HR octadecylsilica 60 Å pore size, 6μM particle size, Waters) contained in a Waters Radial Compression Module eluted with a linear tetrahydrofuran (THF/0,05 M aqueous acetic acid gradient increasing from 15% THF to 30% THF over a period of forty minutes at a flow rate of 15 ml/minute. The eluate was monitored at 320 nm and the fractions containing the peak at retention time 32-37 minutes were collected and extracted with ethyl acetate.

This ethyl acetate extract was concentrated, redissolved in THF/0.05 M aqueous acetic acid (1:1, 6 ml) and then further purified by preparative reverse phase hplc on the same PrepPak octadecylsilica column eluted isocratically with THF/0,05 M aqueous acetic acid (3:7) at a flow rate of 12 ml/minute. The eluate was monitored at 400 nm and that containing the peak at 12-15 minutes was collected and extracted with ethyl acetate. The ethyl acetate extract was concentrated to dryness and redissolved in methanol/0.05 M aqueous acetic acid (1:1, 6.0 ml).

This solution was further purified by a second isocratic reverse phase hplc step using the same PrepPak column eluted with methanol/0.05 M aqueous acetic acid (45:55) at a flow rate of 12 ml/minute. The eluate from the centre of the peak at retention time 10-12 minutes was collected and extracted with ethyl acetate. The ethyl acetate extract was concentrated to dryness to yield a yellow solid (29 mg) of the present compound.

Example 4 - Detection of Compound [A] by Analytical High Performance Liquid Chromatography (hplc)

Partially purified fractions from the first preparative reverse phase hplc fractionation of the crude extract of the fermentation of strain X8063, described in Example 3, were examined for the presence of the desired compound by analytical reverse phase hplc. The analysis was carried out on an hplc system comprising a Waters 600E Multisolvent Delivery System, a Waters U6K Injector and a

Waters 990 Photodiode array detector using a Waters Radial Pak cartridge (8mm ID x 100mm length, packed with Nova-Pak octadecylsilica, 60 Å pore size, 4μM particle size, Waters) contained in a Waters Radial Compression Module eluted isocratically with THF/0.05M aqueous acetic acid (3:7) at a flow rate of 2 ml/minute. The present compound was identified by its UV/visible spectrum using the Photodiode array detector (λmax under acidic conditions: 365 ± 4 nm, 316 ± 3nm, 240 nm (sh)). It appeared as a peak typically having a retention time in the range 7-10 minutes.

Compound [A] has the following ultraviolet (UV), infrared (ir), nuclear magnetic resonance (nmr) and mass spectral (MS) characteristics:

UV: λ^{MeOH}_{max} ($\log_{10}E$) nm: 402(4.17); 319(4.09); 225 sh(438)

UV: $\lambda^{MeOH-0.1MHCl}_{max}$ ($\log_{10}E$) nm: 365(4.04); 316(4.21); 240 sh(4.30)

UV: $\lambda^{MeOH-0.1MNaOH}_{max}$ ($\log_{10}E$) nm: 400(4.21); 345 sh(4.06); 240 sh(4.37)

ir: $\nu^{KBr\ (diffuse\ reflectance)}_{max}$ cm^{-1}: 3200 (br), 1709, 1659, 1603, 1470, 1281, 1200, 1042, 1001, 938, 862

The ^{1}H and ^{13}C NMR spectra of compound [A] consist of overlapping signals from the two isomeric forms of compound [A]:

^{1}H nmr: δ (400 MHz, $(CD_3)_2SO$) ppm:
11.55 (br s), 11.15 (br s), 9.32 (br s), 8.55 (1H, s, form 1), 7.96 (1H, s, form 2), 6.95 (1H, s, form 2), 6.93 (1H, s, form 1), 6.44 (1H, s, form 1), 6.40 (1H, s, form 2), 5.04 (1H, dd, J=4.8, 8.8 Hz, form 1), 4.7 (2H, br s, form 2; 2H, m, form 1), 2.72 (3H, s, form 2), 2.68 (3H, s, form 1), 2.32 (3H, s, form 2), 2.300 (3H, s, form 1).

^{13}C nmr: δ (400 MHz, $(CD_3)_2SO$) ppm:
202.8, 202.5, 199.0, 186.2, 172.8, 172.6, 167.7, 167.4, 156.3, 155.7, 154.6, 154.5, 153.9, 152.2, 151.7, 150.3, 150.2, 140.9, 140.8, 139.2, 138.7, 138.3, 137.6, 134.9, 132.4, 131.9, 127.8, 125.9, 122.3, 121.7,

120.7, 118.7, 118.3, 110.2, 110.1, 109.3, 108.8, 104.9, 102.8, 102.4, 67.0, 65.9, 56.3, 32.4, 32.3, 17.0, 16.8.

ir: KBr (diffuse reflectance): max 3200(br); 1709, 1659, 1603, 1470, 1281, 1200, 1042, 1001, 938, 862

MS: m/z[FAB, MNBA]: 579 [MH^+], 561[$(MH-H_2O)^+$], 543[$(MH-2H_2O)^+$]
m/z[DCI, NH_3]: 535[$(MH-CO_2)^+$], 491[$(MH-2CO_2)^+$]

The accurate mass of compound [A] was determined by mass spectrometry. The ion at m/z 579 in the FAB/MNBA spectrum was measured:

observed mass = 579.0790
(average of two measurements)

calculated mass for $C_{28}H_{19}O_{14}$ = 579.0775

Example 5 - Purification of compound [C]

A portion (8.4g) of the ethyl acetate extract prepared in Example 2 was dissolved in methanol (40ml) and diluted with 0.05 M aqueous acetic acid (40ml). This solution was purified by preparative reverse phase hplc on a PrepPak cartridge column (1D 25 mm x length 100 mm, packed with Nova-Pak HR octadecylsilica 60 Å pore size, 6 μM particle size, Waters) contained in a Waters Radial Compression Module eluted with a linear tetrahydrofuran (THF)/0,05 M aqueous acetic acid gradient increasing from 15% THF to 30% THF over a period of forty minutes at a flow rate of 15 ml/minute. The eluate was monitored at 360 nm and the fractions containing the peak at retention time 31-37 minutes were collected and extracted with ethyl acetate.

This ethyl acetate extract was concentrated to give a brown solid (1.02g). This was redissolved in methanol/0.05 M aqueous acetic acid (1:1, 50 ml) and then further purified by preparative reverse phase hplc on the same PrepPak octadecylsilica column eluted isocratically with

THF/0,05 M aqueous acetic acid (3:7) at a flow rate of 16 ml/minute. The eluate was monitored at 320 nm and that containing the peak at 10-14 minutes was collected and extracted with ethyl acetate. The ethyl acetate extract was concentrated to dryness and to give a yellow-brown solid (252 mg) which was redissolved in methanol/0.05 M aqueous acetic acid (1:1, 15 ml).

This solution was further purified by a reverse phase hplc step using the same PrepPak column eluted with a methanol-0.05M aqueous acetic acid gradient, starting at 45% methanol for the first eight minutes and then increasing linearly from 45 to 55% methanol over the next two minutes, at a flow rate of 18 ml/minute. The eluate was monitored at 320 nm and the eluate from the centre of the peak at retention time 6.5-9 minutes was collected, diluted 1:1 with 0.05M aqueous acetic acid and extracted with ethyl acetate. The ethyl acetate extract was concentrated to dryness to yield a yellow-brown solid (35 mg). This was redissolved in methanol-0.05M aqueous acetic acid (1:1, 7ml) and purified further by isocratic reverse phase hplc using the same PrepPak column eluted with methanol-0.05M aqueous acetic acid (45:55) at a flow rate of 18 ml/minute.

The eluate was monitored at 325 nm and that containing the peak at retention time 8.0 to 9.2 minutes was collected, diluted 1:1 with 0.05M aqueous acetic acid and extracted with ethyl acetate. The ethyl acetate extract was concentrated to dryness and redissolved in methanol-0.05M aqueous acetic acid (1:1, 6ml). This sample was subjected to another isocratic reverse phase hplc purification step using the same PrepPak column eluted with methanol-0.05M aqueous acetic acid (45:55) at a flow rate of 18 ml/minute. The eluate was again monitored at 325nm and that containing the peak eluting from 7 to 9 minutes was collected, diluted 1:1 with 0.05M aqueous acetic acid and extracted with ethyl acetate. The ethyl acetate

extract was concentrated to dryness to yield a brown solid (5.2 mg) of the present compound.

Example 6 - Detection of Compound [C] by Analytical High Performance Liquid Chromatography (hplc)

Partially purified fractions from each of the preparative reverse phase fractionations described in Example 5, were examined for the presence of the desired compound by analytical reverse phase hplc. The analysis was carried out on an hplc system comprising a Waters 600E Multisolvent Delivery System, a Waters U6K Injector and a Waters 990 Photodiode array detector using a Waters Radial Pak cartridge (8mm ID x 100mm length, packed with Nova-Pak octadecylsilica, 60 Å pore size, 4μM particle size, Waters) contained in a Waters Radial Compression Module eluted isocratically with methanol-0.05M aqueous acetic acid (1:1) at a flow rate of 2 ml/minute. The present compound was identified by its UV/visible spectrum using the Photodiode array detector (λmax under acidic conditions: 322 ± 3nm, 287 ± 5nm, 206 ± 3nm). It appeared as a peak typically having a retention time in the range 3.5-4.5 minutes.

Compound [C] has the following UV, ir, nmr and MS characteristics:

UV:	λ^{MeOH}_{max}	$(\log_{10}E)$nm:	381(4.05), 285(4.12), 230(4.40), 208(4.45)
UV:	$\lambda^{MeOH-0.1M\ HCl}_{max}$	$(\log_{10}E)$nm:	321(4.11), 285(4.18), 205(4.45)
UV:	$\lambda^{MeOH-0.1M\ NaOH}_{max}$	$(\log_{10}E)$nm:	440_{sh}(3.78), 345(4.21), 300_{sh} (4.16), 250_{sh} (4.35), 226(4.41)
ir:	$\nu^{KBr\ (diffuse\ reflectance)}_{max}$		cm^{-1}: 3427-3053, 1692, 1659, 1621, 1566, 1518, 1468, 1434_{sh}, 1358, 1291, 1228, 1196, 1163, 1024, 990

^{1}Hnmr: δ(400MHz, $(CD_3)_2SO$) ppm:
8.56 (1H,s), 8.13 (1H,s), 7.48 (1H,s), 6.96 (1H,s), 6.94 (1H,s), 2.54 (3H,s) 2.53 (3H,s)

The ^{13}C nmr spectrum of compound [C] showed signals from twenty five carbon atoms. Signals from two acetyl carbonyl carbons expected at approximately 200 ppm were not observed due to poor signal-to-noise ratio.

^{13}C nmr: δ(400MHz, $(CD_3)_2SO$) ppm:
173.3, 172.9, 167.3, 154.3, 152.6, 152.5, 152.4, 150.7, 150.6, 144.5, 141.7, 136.0, 133.5, 132.3, 126.1, 120.8, 119.7, 119.5, 113.4, 112.4, 108.7, 103.0, 102.3, 32.0, 29.0

MS: m/z [Fast atom bombardment (FAB), m-nitrobenzylalcohol (MNBA)]:
533 [MH^+], 515[$(MH-H_2O)^+$], 489[$(MH-CO_2)^+$]
mz [FAB, MNBA (negative ion)]:
531 [$(M-H)^-$], 513 [$(M-H-H_2O)^-$].
487 [$(M-H-CO_2)^-$]

<u>Example 7 - Culture of strain X8063 in Liquid Media (2)</u>

Starting material of the strain X8063 was generated by suspending a mature slant culture, grown on PDA (2% dextrose, 15% agar, 0.4% potato extract), in 5 ml 10% aqueous glycerol. 1 ml of this suspension, in a 1.5 ml cryovial, comprises starting material, which was retrieved from storage at -135°C. A preculture was produced by aseptically placing 0.5 ml starting material in 20 ml nutrient solutions (1.5% glycerol, 1.5% soya bean peptone, 1% glucose, 0.5% malt extract, 0.3% NaCl, 0.1% $CaCO_3$, 0.1% Tween 80, 0.1% Junlon PW110, pH6) in a test tube shaken at 240 rpm for 2 days at 25°C.

Following this incubation period, 20 ml of the above nutrient solution S was aseptically added to the above preculture and the new mixture was incubated for a further 2 days at 25°C, forming the secondary growth stage. A tertiary growth stage was then produced by aseptically transferring the secondary preculture to 250ml of nutrient solution S in a 2l aspirator and stirring at 500 rpm for 3

days at 25°C.

A production culture was generated by aseptically transferring a tertiary culture to a 141 stirred fermenter containing 101 of nutrient solution P (6.04% molasses. 0.339% casein enzymatic hydrolysate, 0.1% $CaCO_3$, 0.1% Tween 80, 0.006% sodium phytate, pH6) and incubating at 25°C. After 5 days the liquor was harvested for extraction of the product.

Example 8 - Extraction of compound [B] from fermentation of the strain X8063

A 101 fermentation of the strain X8063 prepared as in Example 7 was centrifuged and the mycelium discarded. The pH of the aqueous supernatant was adjusted from 6.5 to 3.0 with glacial acetic acid and then the acidified supernatant was extracted with ethyl acetate (2x4.51). The ethyl acetate extract (6.51) was then back-extracted with 0.1M aqueous ammonium acetate (3x1.61). The pH of the first, aqueous back extract was adjusted from 4.9 to 2.6 by adding concentrated orthophosphoric acid and the acidified back extract was then extracted with ethyl acetate (2x1.01). This ethyl acetate extract was concentrated to dryness (3.8g) and redissolved in methanol (19ml). The second aqueous back extract (pH5.5) was acidified, extracted and concentrated in the same way to give more material (1.5g) containing a little more of the compound of interest.

The methanolic solution of the concentrated first back extract material was purified by preparative reverse phase hplc on a Nova Pack octadecyl silica column (ID 25mm x length 100mm), contained in a Radial Compression Module, eluted with a linear tetrahydrofuran (THF)/0.5M aqueous acetic acid gradient increasing from 15% THF to 30% THF over a period of 40 minutes at a flow rate of 15ml/min. The eluate was monitored at 350nm and the fractions

containing the peak at retention time 23-27 minutes were collected and extracted with ethyl acetate.

This ethyl acetate extract was concentrated, redissolved in THF/0.05M aqueous acetic acid (1:3, 20ml) and then further purified by preparative reverse phase hplc on the same Nova Pak octadecyl silica column eluted isocratically with THF-0.05M aqueous acetic acid (2:8) at a flow rate of 15ml/min. The eluate was monitored at 350nm and that containing the peak at retention time 20-21 minutes was collected and extracted with ethyl acetate. The ethyl acetate extracted was concentrated to dryness to yield crude 1 (40 mg).

Example 9 - Purification of compound [B]

The crude material from Example 8 was subjected to a second purification by high performance liquid chromatography using the column and isocratic solvent system described in Example 8. The eluate from the centre of the peak at retention time 20-21 minutes was collected and extracted with ethyl acetate. The ethyl acetate extract was concentrated to dryness to yield a yellow solid (21mg) of compound [B]. Compound [B] has the following UV, ir, nmr and MS characteristics:

UV: λ_{max}^{MeOH} (log E) nm: 383 (4.11); 315 (4.12); 275 (4.38); 225 (4.83)

UV: $\lambda_{max}^{MeOH-CH_3CO_2H}$ (log E) nm: 322 (4.19); 286 (4.24); 237 (4.36)

UV: $\lambda_{max}^{MeOH-NaOH}$ (log E) nm: 356 (4.08); 284 (3.97); 225 (4.28)

ir: ν_{max}^{KBr} (diffuse reflectance) cm^{-1}: 3300 (br), 1696, 1615, 1570, 1464, 1358, 1289, 1223, 1172, 936

^1H nmr:	δ(500 MHz, $(CD_3)_2SO$) ppm: 12.7 (2H, br s), 11.7 (2H, br s), 9.4 (2H, br s), 8.54 (1H, s), 8.18 (1H, s), 6.96 (1H, s) 6.95 (1H, s), 2.56 (3H, s), 2.55 (3H, s).
^{13}C nmr:	δ(270MHz, $(CD_3)_2SO$) ppm: 201.2, 199.2, 172.9, 172.6, 167.4(2C), 154.1, 152.8, 152.6, 152.2, 150.8, 150.4, 141.8, 141.1, 136.4, 133.5, 132.7, 126.5, 121.0, 120.7, 119.9, 119.7, 112.6, 110.0, 102.4, 102.3, 32.3, 29.2
MS:	m/z[DCI, NH_3]: 577 [MH^+], 533 [$(MH-CO_2)^+$], 489 [$(MH-2CO_2)^+$] m/z [Fast atom bombardment (FAB), m-nitrobenzyl alcohol (MNBA)]: 577 [MH^+], 559 [$(MH-H_2O)^+$], 541 [$(MH-2H_2O)^+$]

The accurate mass of compound [B] was determined by mass spectrometry. The ion at m/z 577 in the FAB/MNBA spectrum was measured:

observed mass = 577.0730

calculated mass for $C_{28}H_{17}O_{14}$ = 577.0612

Example 10 - Detection of Compound [B] by Analytical High Performance Liquid Chromatography (hplc)

Partially purified fractions from the preparative reverse phase fractions described in Example 9 were examined for the presence of the desired compound by analytical reverse phase hplc. The analysis was carried out on an hplc system comprising a Waters 600E Multisolvent Delivery System, a Waters U6K Injector and a Waters 990 Photodiode array detector using a Waters Radial Pak cartridge (8mm ID x 100mm length, packed with Nova-Pak

octadecylsilica, 60 Å pore size, 4μM particle size, Waters) contained in a Waters Radial Compression Module eluted isocratically with methanol-0.05M aqueous acetic acid (4:6) at a flow rate of 2 ml/minute. The present compound was identified by its UV/visible spectrum using the Photodiode array detector (λmax under acidic conditions: 322 ± 4 nm 286 ± 4 nm, 237 ± 4 nm). It appeared as a peak typically having a retention time in the range 4-6 minutes.

Example 11 - Methylation of Compound [B]

Compound [B] (5 mg) was dissolved in tetrahydrofuran (2.5 ml) and treated with an excess of a solution of diazomethane in ether generated by the action of concentrated aqueous potassium hydroxide on Diazald reagent (N-methyl-N-nitroso-p-toluene sulphonamide, Aldrich Chemical Co. Ltd). After 15 minutes the reaction mixture was rotary evaporated to dryness to yield a brown solid which was identified as the permethylated derivative of compound [B] from its mass spectra:

MS: m/z [Desorption Chemical Ionisation (DCI), NH_3]:
661 [MH^+]
m/z [Electron impact, EI]:
645 [$(M-CH_3)^+$], 629 [$(M-OCH_3)^+$],
617 [$(M-CH_3CO)^+$]

Example 12 - Assay for PKC inhibitory activity

PKC-inhibitory activity was quantified by the ability of a compound to decrease phosphorylation of a PKC-specific substrate by the enzyme. The assay employed PKC purified from bovine brain, necessary cofactors for enzyme activity (calcium, phospholipid and phorbol ester), a peptide substrate derived from a naturally-occurring PKC phosphorylation site in the EGF receptor and the donor substrate [$\gamma^{32}P$]ATP.

An incubation mixture of 75μl containing Ca^{2+},

micelles of phosphatidylserine/phorbol ester, peptide substrate and inhibitor was preincubated at 25°C for 5 minutes before the reaction was initiated by the addition of 10μl [γ^{32}P]ATP mixture (0.04 μCi/assay). Following 2h incubation at 25°C, the reaction was terminated by the addition of 50μl dilute orthophosphoric acid. Aliquots of 100μl were spotted onto phosphocellulose paper squares (1 cm x 1 cm) and the binding papers placed in 75 mM orthophosphoric acid, at least 10 ml of this wash reagent being allowed per paper. After 20 minutes with intermittent gentle mixing, the wash reagent was replaced with a similar volume of fresh wash reagent. Following a further 20 minute wash period, the papers were placed into individual scintillation vials and counted for ^{32}P in a scintillation counter.

The ^{32}P incorporated into peptide was quantitatively measured by the binding papers. The results obtained were corrected for any non-specific effect using appropriate blanks (no enzyme, no substrate). The inhibition of PKC activity was determined using the following equation:

$$\% \text{ inhibition} = \frac{\text{Non-inhibited control cpm} - \text{sample cpm}}{\text{Non-inhibited control cpm}} \times 100\%$$

The following Tables 8 and 9 show the degree of inhibition absorbed at various concentrations:

TABLE 8: Inhibition of PKC by compounds [A] and [B]

Concentration of compound, $Mx10^6$	% Inhibition [A]	% Inhibition [B]
0.01	8	-
0.05	-7	-5
0.1	13	2
1	31	40
10	65	77
50	72	76
100	77	73
200	80	71

TABLE 9: Inhibition of PKC by compound [C]

Concentration of compound, $Mx10^6$	% Inhibition
0.6	- 27
0.56	29
5.6	51
54.5	72
553	91

Example 13 - Pharmaceutical composition

Tablets, each weighing 0.15g and containing 25mg of the present compound can be manufactured as follows:

Composition for 10,000 tablets

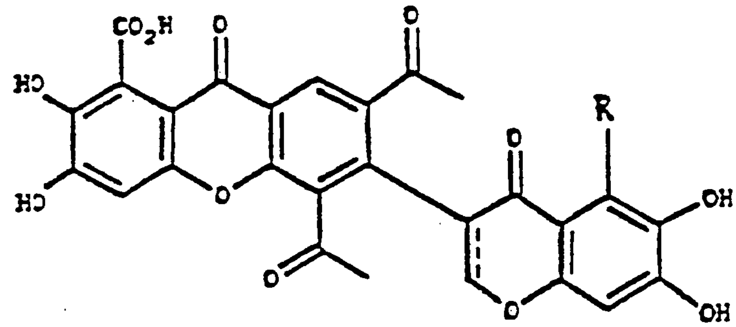
Present compound (250g)
lactose (800g)
corn starch (415g)

talc powder (30g)
magnesium stearate (5g)

The present compound, lactose and half the corn starch are mixed. The mixture is then forced through a sieve 0.5mm mesh size. Corn starch (10g) is suspended in warm water (90ml). The resulting paste is used to granulate the powder. The granulate is dried and comminuted on a sieve of 1.4mm mesh size. The remaining quantity of starch, talc and magnesium stearate is added, carefully mixed and processed into tablets.

CLAIMS

1. A xanthone derivative having the formula 1:



wherein ---- is a single bond and R is -COOH or ---- is a double bond and R is H or -COOH; and pharmaceutically and veterinarily acceptable salts, esters and ethers thereof.

2. A compound according to claim 1 which is selected from
2,4-diacetyl-3-(2',3'-dihydro-5'-carboxy-6',7'-dihydroxychromon-3'-yl)-6,7-dihydroxyxanthone-8-carboxylic acid;
2,4-diacetyl-3-(5'-carboxy-6',7'-dihydroxychromon-3'-yl)-6,7-dihydroxyxanthone-8-carboxylic acid and the permethylated derivative thereof; and
2,4-diacetyl-3-(6',7'-dihydroxychromon-3'-yl)-6,7-dihydroxyxanthone-8-carboxylic acid.

3. A process for the preparation of a xanthone derivative as defined in claim 1 or a pharmaceutically or veterinarily acceptable salt, ester or ether thereof, which process comprises (i) fermenting, in a source of carbon, nitrogen and inorganic salts, fungal strain X8063 (CMI

336456) or a mutant thereof which produces a said xanthone derivative; (ii) isolating a said xanthone derivative from the fermentation medium; and (iii) if desired, converting the said xanthone derivative into a pharmaceutically or veterinarily acceptable salt, ester or ether thereof.

4. A compound as claimed in claim 1 for use in a method of treatment of the human or animal body by therapy.

5. A compound according to claim 4 for use as a CD4 binding agent.

6. A compound according to claim 5 for use in the treatment of an autoimmune disease, graft versus host disease or organ rejection.

7. A compound according to claim 6 for use in the treatment of rheumatoid arthritis, systemic lupus erythematosus, diabetes mellitus, multiple sclerosis or primary biliary cirrhosis.

8. A compound according to claim 5 for use in the treatment of allergic diseases and inflammatory diseases.

9. A compound according to claim 5, for use as an anti-HIV agent.

10. A compound according to claim 4 for use as an inhibitor of Protein Kinase C.

11. A compound according to claim 4, for use as a collagenase inhibitor.

12. A compound according to claim 10 or 11 for use as an antitumour agent, an antiinflammatory agent, an agent for treating autoimmune disease or an immunosuppressive agent.

13. A pharmaceutical or veterinary composition comprising a pharmaceutically or veterinarily acceptable carrier or diluent and, as active ingredient, a compound as claimed in claim 1.

14. <u>Penicillium glabrum</u> X8063 (CMI 336456) or a mutant thereof which produces a xanthone derivative of formula <u>1</u> as defined in claim 1.

15. A biologically pure culture of fungal strain X8063 (CMI 336456) or of a mutant thereof which produces a xanthone derivative of formula 1 as defined in claim 1.

16. A process for fermenting fungal strain X8063 (CMI 336456) or a mutant thereof which produces a xanthone derivative of formula 1 as defined in claim 1, which process comprises fermenting strain X8063 or a said mutant thereof in a source of carbon, nitrogen and inorganic salts.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/00526

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07D311/86; C07D493/14; C12N1/14; C12P17/06
A61K31/35

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07D ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	CHEMICAL ABSTRACTS, vol. 114, no. 19, 13 May 1991, Columbus, Ohio, US; abstract no. 181059S, M. AOKI et al.: 'Structural determination of a novel phospholipase C inhibitor' page 349 ; & Tennen Yuki Kagobutsu Toronkai Koen Yoshishu, 1990, 32, 190-196 ---	1,2,4,12

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 MAY 1992	27 MAY 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	RUSSELL F. ENGLISH Russell F. English

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